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Invited Review

Organophosphate Toxicology: Safety Aspects of Nonacetylcholinesterase Secondary Targets

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1. Introduction

OP¹ esters are one of the most important classes of insecticides with 150 different compounds (62 current and 88 superceded) used for protecting crops, livestock, and human health in the past 60 years (1–3). The OPs and the methylcarbamates (with the same mode of action) accounted for 52% of the insect control chemical

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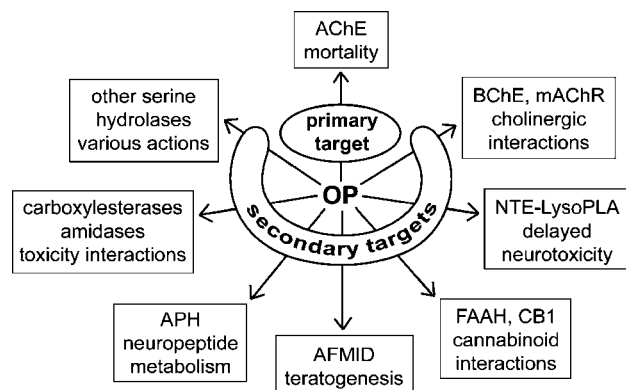


Figure 1. Primary and secondary targets of OP serine hydrolase inhibitors.

world market of about eight billion U.S. dollars in 1999, down from 71% in 1987 (4). Almost every person is or has been exposed to OP insecticides in their home or work environment or as trace dietary contaminants (5). Intoxication by OPs, occasionally leading to death, represents up to 80% of pesticide-related hospital admissions (6). OPs are also major chemical warfare agents with extensive stockpiles as a continuing threat worldwide (7). Selected OPs are used in medicine as anthelmintics and ectoparasiticides and for glaucoma and have been tested for myasthenia gravis and Alzheimer's disease (6, 8). Thus, OPs are essential tools and probes in agriculture and medicine.

The primary target of OP insecticides and chemical warfare agents is AChE as discovered independently in Germany and England in 1940–1942 (9–11). Most safety evaluations of OP insecticides are based on the premise that AChE inhibition is the principal action for both acute and chronic toxicity of individual compounds or mixtures. Clearly, OPs act on non-AChE systems raising questions on the contribution of these alternate targets to the acute lethal action and secondary effects of short- or long-term exposure (12–14) (Figure 1). What are the safety aspects of non-AChE secondary targets?

2. Serine Hydrolase Secondary Targets

Six serine hydrolases and the CB1 receptor are of current interest in considering OP toxicology (Table 1). Four are membrane bound, and three are cytosolic or secreted. They range from 34 to 155 kDa and in number of amino acids from 305 to 1327. AChE and BChE have ACh as their endogenous substrate and a Glu-His-Ser catalytic triad. The third serine hydrolase is a specific LysoPLA first known as NTE and now designated NTE-LysoPLA with lysolecithin as an endogenous sub-

strate and Asp-Asp-Ser as the catalytic triad. Three others are amidases, i.e., FAAH with Ser and Lys at the catalytic site and AFMID and APH with Asp-His-Ser as the triad. Their endogenous substrates are anandamide (or oleamide), *N*-formyl-L-kynurenine, and *N*-acetyl peptides, respectively. All of these hydrolases except FAAH have the typical GX₂SG motif. The enzymes have α/β hydrolase folding except FAAH with the amidase signature sequence. The degree of glycosylation varies with the enzyme and receptor. The CB1 receptor is also inhibited, but the site of block is not defined. Mouse gene deletions have been prepared for AChE, NTE-LysoPLA, FAAH, and CB1, and *Drosophila* gene deletions have been prepared for AChE and the NTE-LysoPLA counterpart. This is only a very small fraction of the total serine hydrolases, which are a large group of enzymes, e.g., 3% of the *Drosophila* genome (33).

The OP sensitivity or reactivity profile (Table 2) differs for each serine hydrolase and the CB1 receptor (individual sensitivities considered later). The OPs and other AChE inhibitors have been examined in a plethora of enzyme and organismal systems in search of actions not attributable to AChE inhibition. Some of the investigations used compounds designed to emphasize non-AChE effects. This also applies to alkanesulfonyl fluorides, e.g., DSF. Many of the studies used mice as mammalian models for toxicological relevance and because of their known genomic background, gene deletion animals, and economy of size in required amounts of compound.

3. Reactions of OP Compounds with Serine Hydrolases

The OP toxicants considered here (Figure 2) are phosphorylating agents (2, 43) as illustrated in Scheme 1 with ChE as an example. They inhibit the hydrolase in a progressive manner by dialkylphosphorylation of the active site Ser of a catalytic triad also involving His, Lys, Glu, or Asp, shown in Figure 3 by the interaction of CPO with AChE. The dialkylphosphorylated enzyme may undergo reactivation (which can be facilitated for AChE by the antidote pralidoxime) or spontaneous aging (loss of an alkyl group) to form the monoalkylphosphoryl-ChE, an essentially irreversibly inhibited enzyme (6). Dialkylphosphoryl- and monoalkylphosphoryl-AChE in subpicomole amounts can be observed directly by matrix-assisted, laser desorption, time-of-flight mass spectrometry using the brain of a single poisoned mouse (44). After inhibition of AChE by mipafox, there is a loss of both isopropylamino groups to give "doubly aged enzyme" (45). Phosphoryl-ChE can be prepared by reaction of AChE at the active site serine with HOP(O)Cl₂ (46, 47) and BChE with ethephon (a plant growth regulator) (48, 49). The facile aging reaction for dialkylphosphoryl-AChE is also applicable to BChE (50). Aging of the mipafox-inhibited catalytic domain of human NTE involves reversible loss of a proton from nitrogen rather than irreversible loss of an isopropylamino substituent (51). Chiral OPs have enantiomeric specificity at each stage of the binding, phosphorylation, aging, and dephosphorylation reactions. On this basis, the individual enantiomers may even have different leaving groups directly or following bioactivation on reaction with the esteratic site (52, 53). AChE and BChE are only two of many serine hydrolases undergoing phosphorylation and dephospho-

¹Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; AFMID, arylformamidase; APH, acylpeptide hydrolase; BChE, butyrylcholinesterase; CB1, cannabinoid CB1 receptor; CD, *cis*-methyl-dioxolane; ChAT, choline acetyltransferase; ChE, cholinesterase; CP, chlorpyrifos; CPO, chlorpyrifos oxon; DFP, diisopropyl fluorophosphate; DSF, dodecanesulfonyl fluoride; EOPF, ethyl octylphosphonofluoridate; ERK, extracellular signal-regulated kinase; FAAH, fatty acid amide hydrolase; HACU, high-affinity choline uptake; IDFP, isopropyl dodecylfluorophosphonate; LysoPLA, lysophospholipase; mAChR, muscarinic acetylcholine receptor; MAFP, methyl arachidonylphosphonofluoridate; nAChR, nicotinic acetylcholine receptor; NMS, *N*-methylscopolamine; NTE, neuropathy target esterase; OBDPO, octyl-4*H*-1,3,2-benzodioxaphosphorin 2-oxide; OP, organophosphorus; OPIDN, organophosphorus-induced delayed neuropathy; OXO, oxotremorine; PKC, protein kinase C; QNB, quinuclidinyl benzilate; TOCP, tri-*o*-cresyl phosphate; vAChT, vesicular acetylcholine transporter; VX, *O*-ethyl *S*-[2-(diisopropylamino)ethyl]methylphosphonothioate.

Table 1. Properties of Six Serine Hydrolases and the CB1 Receptor as Organophosphate-Sensitive Targets

property	AChE	BChE	NTE-LysoPLA	FAAH	CB1	AFMID	APH
E.C. no.	3.1.1.7	3.1.1.8		3.5.1.60		3.5.1.9	3.4.19.1
endogenous assay	ACh	ACh	substrate (ligand)				
	ATCh ^a	BTCh ^a	lysolecithin	anandamide		<i>N</i> -formyl-L-kynurenine	<i>N</i> -Ac peptides AANA ^a
			phenyl valerate	oleamide	(CP 55 940)		
			structure ^b				
membrane bound	yes	no	yes	yes	yes	no	no
kDa	65	85	155	63	60	34	85, 75
amino acids	583	574	1327	579	473	305	732
catalytic triad	S203	S198	S966	S241		S162	S587
	H447	H438	D960	K142		H279	H707
	E334	E325	D1086	S217		D247	D675
GXSG motif folding ^d	GESAG	GESAG	GTSIG	GG SIR ^c		GHSAG	GGSHG
	α/β -hydrolase	α/β -hydrolase	α/β -hydrolase	amidase signature sequence		α/β -hydrolase	α/β -hydrolase
primary and tertiary glycosylation (ref)	15, 16	17, 18	19	20, 21	22	23	24
	yes	yes	yes		yes	no	no
			gene deletion (refs)				
<i>Drosophila</i>	25	no	26	no	no	no	no
mouse	27	no	28–30	31	32	no	no
classical OP inhibitors ^e	VX, paraoxon	CPO, DFP	mipafox, DFP	MAFP, CPO	MAFP, CPO	diazoxon	DFP

^a Abbreviations: acetylthiocholine (ATCh), butyrylthiocholine (BTCh), and *N*-acetyl-Ala-*p*-nitroanilide (AANA). ^b Species: mouse for AChE, CB1, and AFMID; human for BChE, NTE-LysoPLA, and FAAH; and rat and porcine for APH. ^c Analogous motif. ^d On the basis of X-ray crystallography or homology modeling. ^e Structures are given in Figure 2.

Table 2. Organophosphate-Sensitivity Profiles of Six Serine Hydrolases and the CB1 Receptor

OP ^a	IC ₅₀ (nM) ^{b,c}						
	AChE	BChE	NTE-LysoPLA	FAAH	CB1	AFMID	APH
	fluorophosphorus compounds						
DFP	9000	26	1200	48 000	> 10 000	1700	17
mipafox			13 000	> 10 000			61 000
MAFP	124	2	0.6	0.1	530		
EOPF	120	4	0.05	0.6	11 000		210
IDFP	700	55	ca. 0.2	2	2		11 000
	aryl phosphates						
paraoxon	13	7	> 10 ⁵	540	1200	10	12 000
CPO	19	< 1	180	40	14		82
diazoxon					2000	45	
profenofos	5000		2300	270	39 000		180 000
OBDO	300	18	0.1	8	120		
	aliphatic phosphate						
dichlorvos	5100		30 000	1800	4200		2900
	sulfonyl fluoride						
DSF	> 10 ⁵	> 10 ⁵	470	2	7		> 100 000

^a Structures are given in Figure 2, and chemical names are given in the abbreviations footnote. ^b Data from refs 23 and 34–41. APH was determined by the method of Richards et al. (42). All enzymes were from mouse brain except BChE (mouse blood) and AFMID (mouse liver). Most assays involved 15 min of inhibition time at 25 °C. ^c Diacylglycerol lipase (mouse brain) IC₅₀ value was 3000 nM for CPO and 1000 nM for EOPF. Phospholipase A₂ (mouse brain) IC₅₀ value was > 10⁴ nM for CPO, EOPF, IDFP, and DSF.

rylation reactions at their active sites. The reaction kinetics depend on the OP and the hydrolase, i.e., the nucleophilicity, lipophilicity, and stereospecificity of the OP and the positioning and nature of the catalytic triad within the active site.

Many of the targets of the OPs are shared by suitably substituted methylcarbamates and sulfonyl fluorides. The methylcarbamates are major insecticides as substituted phenyl and aldoxime esters (54). The methylcarbamoylated enzyme is much less stable than the phosphorylated enzyme, and the reversibility of AChE inhibition is a significant safety factor for the methylcarbamate insecticides (e.g., carbaryl and aldicarb) (43, 54). None of the sulfonyl fluorides or esters are commercial insecticides since they are not as effective as the OPs. There is no possibility for aging (loss of an alkyl moiety to give an ionized substituent) following sulfonylation, presumably at the active site Ser.

4. ChEs and the Cholinergic System

ACh is an endogenous neurotransmitter in the central and peripheral nervous systems, acting at cholinergic synapses and neuroeffector junctions (55). The cholinergic system consists of components for ACh synthesis, transport, and hydrolysis (AChE and BChE) plus muscarinic receptors (mAChRs) and nicotinic receptors (nAChRs) for signal transduction. nAChRs are ligand-gated ion channels while mAChRs are G-protein-coupled receptors. The OP sensitive sites are AChE, BChE, and mAChR (Figure 1).

4.1. AChE

4.1.1. OP Effects

Mammals and insects are severely poisoned or die when OPs strongly inhibit AChE at critical neuronal sites

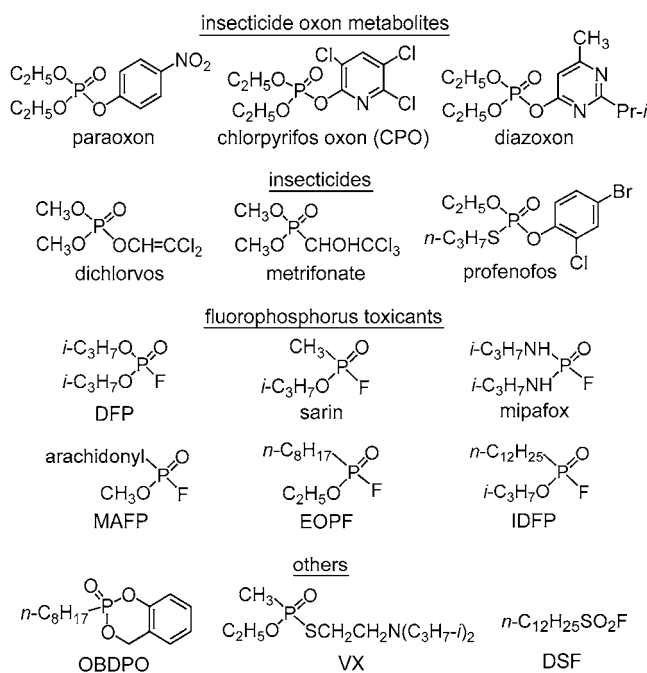


Figure 2. OP insecticides and their metabolites, chemical warfare agents (sarin and VX with DFP as a model), and designer compounds for potency or selectivity (MAFP, EOPF, IDFP, and OBDPO).

(2, 6, 56). The cause of death from OP poisoning of mammals is usually respiratory failure, and fatal doses may not significantly affect brain AChE activity (10). In addition to this acute lethal effect, human survivors of a persistent cholinergic crisis sometimes suffer with an "intermediate syndrome" characterized by delayed muscle weakness (57).

4.1.2. OP Structural Requirements

The insecticides are mostly *O,O*-dimethyl or *O,O*-diethyl phosphates or phosphorothionates (1, 2) (Figure 2). Paraoxon, CPO, and diazoxon are the bioactivated oxon metabolites of the corresponding phosphorothionates parathion, CP, and diazinon. The bioactivation involving P450-catalyzed desulfuration is an important aspect of selective toxicity. The chemical warfare agents are fluorophosphorus compounds or methylphosphonates (DFP, sarin, and VX). Designer compounds (MAFP, EOPF, IDFP, and OBDPO) have also been studied with longer alkyl chains.

4.1.3. Mechanisms of Survival of Mice Lacking AChE

The deletion of AChE in *Drosophila* through mutagenesis results in embryonic lethality (25) as expected from the critical importance of this target. It was therefore expected that gene deletion in mice would result in embryonic death. However, amazingly, *Ache*^{-/-} mice survive over 1 year when fed on a liquid diet, thereby providing a unique model for studies on the cholinergic system and its importance in OP toxicity (27, 58). *Ache*^{-/-} mice suffer from pulsating paws, pinpoint pupils, body tremors, a film of white mucus on the eyes when handled, and lack of grip strength (58, 59). M₁, M₂, and M₄ mAChRs in *Ache*^{-/-} mice are downregulated while the mRNA levels for mAChRs remain unchanged (60); in

Scheme 1. Phosphorylation and Aging of ChE^a

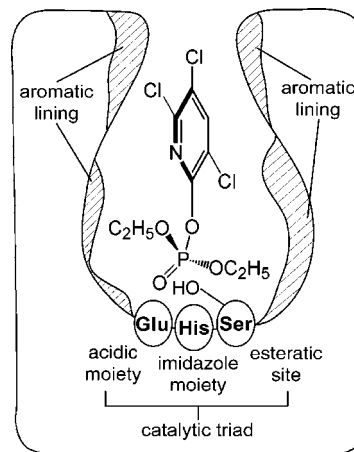
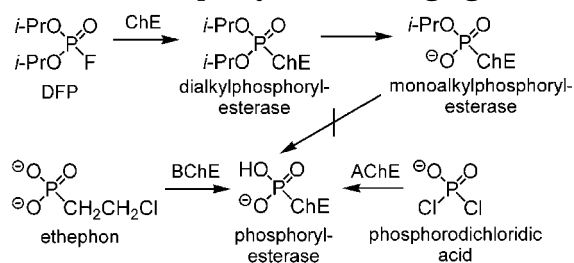


Figure 3. Schematic representation of the interaction of CPO with the AChE active center. The catalytic triad lies near the bottom of a deep and narrow gorge that reaches halfway into the protein. The formation of the diethylphosphoryl derivative on the serine residue involves the imidazole and acidic moieties. The aromatic lining orients the inhibitor and serves as an "aromatic guidance mechanism" for the positive charge on the nitrogen atom of ACh. On the basis of Bourne et al. (16) and other crystal structures and on Lotti (11).

comparison, repeated exposure of rats to OP insecticides decreases mAChRs but differentially regulates mRNA levels for the subtypes (61). Although M₂ receptors are primarily found in neuronal membranes in wild-type mice, in AChE deficient mice, they are principally concentrated in the Golgi complex and endoplasmic reticulum (62); the *Ache*^{-/-} mice are resistant to pilocarpine- and OXO-induced symptoms but more sensitive to atropine (60). Both acute loss of AChE activity by inhibition with metrifonate and chronic loss through deletion of the *Ache* gene result in a dramatic decrease in cell surface somatodendritic M₂ mAChR; however, the M₂ density is higher at the membrane of cortical varicosities in *Ache*^{-/-} mice but unchanged in acutely AChE-inhibited mice (63). Furthermore, mAChR agonist-induced activation of ERK, a signaling pathway associated with synaptic plasticity and amyloidogenesis, is reduced in the hippocampus and cortex of *Ache*^{-/-} mice (64). These mice have no alterations in dopamine receptors or the β 2 subunit of the nAChRs (65). In the striatum, ChAT activity and vesicular ACh transporter (vAChT) levels remain unchanged, but the HACU transporter level is upregulated by 60% in the AChE deficient mice perhaps in response to decreased synaptic ACh metabolism (65).

BChE is an obvious candidate to take over in mice lacking AChE. BChE activity is the same in various

tissues of wild-type and nullizygous mice (66). Although BChE is primarily glial in origin (67), it is also abundant in normal neuromuscular junctions (68). Therefore, BChE may hydrolyze excess ACh at the postsynaptic membrane or may be involved in a presynaptic modulatory step of the ACh release process. BChE decreases presynaptic ACh release contributing to survival (68). However, AChE deficient mice still suffer from cholinergic symptoms, implying that there is not enough ACh being hydrolyzed. It appears that BChE is essential to survival peripherally but not centrally in *Ache*^{-/-} mice (69). Thus, the survival of the *Ache*^{-/-} mice can be attributed to a carryover function of peripheral BChE activity and/or downregulation of cholinergic receptors (also see Section 4.2).

4.1.4. Mechanisms of OP Toxicity in Mice Lacking AChE

Ache^{-/-} mice are not only sensitive to DFP and VX but are even more sensitive than wild-type mice (27, 59). VX gives the traditional signs associated with OP administration beyond the symptoms of the untreated AChE deficient mouse, i.e., loss of motor activity, flattened posture, peripheral vasodilation, more severe whole body tremors, hypothermia, salivation, urination, and tonic convulsions (59). These observations appear at first to be inconsistent with AChE as the primary target of OP toxicants. Furthermore, they indicate that inhibition of targets other than AChE by OPs results in death (27). The specific BChE inhibitor bambuterol at 50 µg/kg sc is lethal to *Ache*^{-/-} mice without any effect on *Ache*^{+/+} mice, in each case inhibiting plasma but not brain BChE activity, indicating the involvement of peripheral BChE inhibition in poisoning (27, 69). Zebrafish mutants lacking AChE (point mutation) and BChE (absent naturally from this genome) suggest secondary targets of AChE inhibitors (70).

4.2. BChE

BChE was the first recognized secondary target in OP poisoning and continues to be of interest as a way to monitor OP exposure by determining the inhibition of plasma activity. It is generally more sensitive than AChE to OP esters (Table 2). BChE is also the most sensitive enzyme to ethephon, which undergoes inhibition in vitro and in vivo forming phosphoryl-BChE (Scheme 1) (48, 49). Ethephon inhibits plasma BChE in subchronic studies with mice, rats, dogs, and humans with no apparent ill effects (48, 49). Animals with inhibited BChE are of enhanced sensitivity to succinylcholine, mivacurium, and aspirin (71). The potency of cocaine in humans and lethality in mice is enhanced by OP pesticide inhibition of BChE (72, 73). BChE as a detoxifying enzyme administered iv decreases the lethality of sarin and the toxicity of cocaine (74, 75). Because the physiological effects of cocaine are conferred by the (-)-isomer, which is hydrolyzed in plasma 2000-fold slower than the (+)-isomer, current research emphasizes understanding the mechanism for cocaine hydrolysis and engineering of catalytically more active BChE (75–77). The functions of BChE will be better understood when it is possible to study BChE deficient mice, but it is already evident that it serves as a possible peripheral target in OP poisoning

(see Section 4.1) and as a scavenger in reducing OP toxicity.

4.3. Muscarinic Receptors

mAChRs are G-protein-coupled receptors of five subtypes: M₂ and M₄ linked to inhibition of adenylyl cyclase and M₁, M₃, and M₅ coupled to stimulation of phospholipase C and phosphoinositide turnover (55). The M₂ mAChRs are the primary subtypes in the heart regulating muscle contraction. Gene deficient mice show that the M₂ mAChR plays a key role in cardiac function, mediating mAChR-dependent movement, temperature control, and analgesia (78). The muscarinic effects of ACh can be blocked by atropine, which thereby serves as an antidote for OP poisoning. Specific binding of [³H]CD and [³H]OXO directly measures the M₂/M₄ agonist site whereas the mAChR antagonists [³H]QNB and [³H]NMS bind nonselectively to all subtypes.

4.3.1. OP Effects in Vitro

Rat heart M₂ and brain mAChRs are sensitive to many OP anticholinesterases at 1–100 nM (79–83). Thus, soman, VX, sarin, and tabun at 0.8–50 nM inhibit [³H]CD binding at the rat M₂ cardiac mAChR (79) and paraoxon at 80 nM inhibits [³H]CD binding in rat brain striatum (80). The sensitivity and specificity of inhibitors are very similar for inhibition of rat brain [³H]CD binding and AChE activity (84). CPO inhibits binding of [³H]CD to M₂/M₄ mAChRs in rat frontal cortex (*K*_i = 2 nM) and inhibits the coupled cAMP formation (*IC*₅₀ = 56 nM) (81) with similar findings in rat striatum (82). CPO and paraoxon directly inhibit cAMP formation through the mAChR in 7 day old rats (*IC*₅₀ = 15–52 nM) (83), and these OPs can activate muscarinic autoreceptors indirectly by inhibition of AChE, thereby mediating alterations in ACh release (85). CPO displaces [³H]OXO binding to rat M₂ cardiac mAChR (*IC*₅₀ = 7–15 nM) (86). Paraoxon modulates signal transduction as [³H]NMS binding in human SK-N-SH and SH-SY5Y neuroblastoma cells partly by indirect activation of mAChRs as well as by acting at an unknown distal site along the pathway (87, 88). The rat heart M₂ mAChR is phosphorylated by [³H-ethyl]CPO in vitro at an unknown site (89).

4.3.2. OP Effects in Vivo

OPs have several mAChR-mediated effects in vivo in rats or guinea pigs. CP at subchronic doses (30 mg/kg sc, four weekly treatments) decreases [³H]CD (but not [³H]QNB) binding to mAChR in rat brain striatum when AChE is inhibited 70% but in the absence of overt clinical signs (90). At 3 mg/kg daily by gavage, CP reduces nerve growth factor levels and the forebrain density of mAChR in rat pups, measured as [³H]QNB binding (91). At 1 mg/kg sc in newborn rats, it induces hepatic adenylyl cyclase activity (92). At gestational or postnatal doses below the threshold for AChE inhibition, CP elicits developmental neurotoxicity, possibly by disruption of the adenylyl cyclase signaling cascade (93, 94). CP (70 or 390 mg/kg, sc) in guinea pigs causes airway hyperreactivity in the absence of AChE inhibition by decreasing the neuronal M₂ receptor function (95). CP at the maximum tolerated sc dose (>85% AChE inhibition) reduces QNB and OXO binding up to 55% in rat brain (96, 97). Chronic

administration of disulfoton at 10 mg/kg/day (ip) gives a loss of mAChRs measured as [³H]QNB binding (98) and at 2 mg/kg/day (oral) differentially regulates mRNA levels of mAChR subtypes in different brain areas (61). Repeated low-dose exposure to methyl parathion (0.3–0.6 mg/kg oral daily) reduces the density of [³H]NMS and [³H]QNB binding sites (99). In summary, the heart and brain M₂/M₄ mAChRs are sensitive to OPs in vitro and in vivo by direct action and as an indirect response to elevated ACh levels. The toxicological relevance of these muscarinic effects is difficult to interpret because of coupling to complex signal transduction systems.

4.4. Other Cholinergic Components

Various OPs act in the presynaptic membrane, through direct inhibition or transcriptional regulation, to affect ChAT, HACU, and vAChT. HACU as assayed by hemicholinium binding is the rate-limiting step for ACh synthesis by ChAT and is responsive to nerve impulse activity. CP administered during gestation and postnatally at doses showing no overt toxic signs (1–7 mg/kg, daily, oral or sc) results in differential changes in ChAT and reductions in HACU activities in adolescent and adult rat brains (100–103). In adult rats treated sc, CP at 280 mg/kg and parathion at 7 mg/kg reduce striatal synaptosomal HACU at 1–7 days after exposure (104, 105). CPO and paraoxon are poor in vitro inhibitors (IC₅₀ > 200 μM) of HACU in rat striatum (104, 105), and DFP is only a moderately potent inhibitor in rat iris (106). In sagittal mouse brain slices, DFP (1 μM) in vitro induces overexpression of the immediate early response transcription factor c-Fos (10 min) with a consequent 8-fold increase in AChE mRNA and a 3-fold decrease in the mRNA encoding ChAT and vAChT (20 min) (107). CP and parathion have age-related effects on ACh synthesis in rat striatum (108).

Nicotinic and muscarinic autoreceptors are involved in ACh release by enhancement and inhibition, respectively (109). CPO at 1–10 μM inhibits nicotinic autoreceptor function in rat brain synaptosomes, and CP at 279 mg/kg (sc) reduces autoreceptor function by 67–91% 96 h after administration in adult and aged rats without affecting juveniles, although AChE inhibition was similar in all groups (109). The OP concentrations required to act directly on nAChRs are much higher than those on mAChRs (110), suggesting that the nAChRs are not important as secondary targets in OP action.

5. Lipases, Signal Transduction, and Gene Expression

Lipids are important structural components and signal transducers. The signaling molecules are often esters, which are made and destroyed as required to synchronize critical functions. The destruction usually involves serine hydrolases. The transducers signal protein kinase systems to phosphorylate and activate proteins, which are then dephosphorylated and deactivated by protein phosphatases. The kinase and phosphatase reactions are generally not OP toxicant sensitive. Glycerol ester signaling molecules are of particular current interest.

5.1. NTE-LysoPLA

5.1.1. OP Effects

A serious problem of OP poisoning was recognized in 1930 when more than 10 000 people developed delayed,

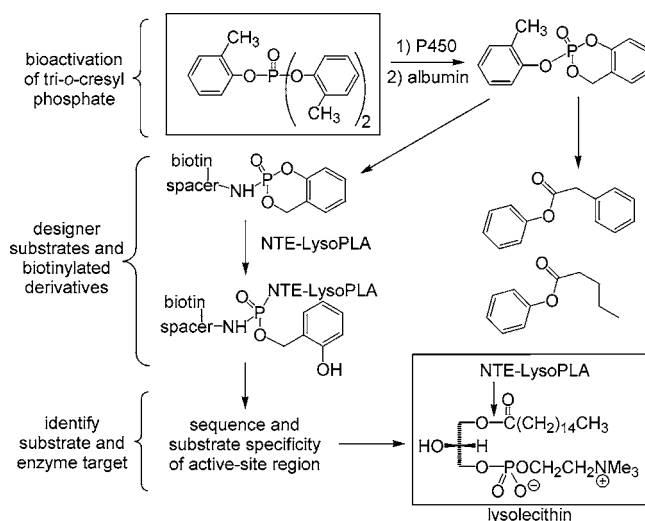


Figure 4. Three steps in defining the mechanism of organophosphate-induced delayed neuropathy first observed with TOCP. Other critical observations were the labeling of the target esterase by [³H]DFP and the aging of the diisopropylphosphoryl enzyme. NTE-LysoPLA is the mipafox sensitive and paraoxon resistant portion of the total esterase activity or [³H]DFP labeling.

irreversible peripheral neuropathy, and the origin was traced to TOCP (Figure 4) (111, 112); a second incident in Morocco in 1959 and others led to over 10 000 more cases of TOCP-induced polyneuropathy (113, 114). TOCP is of continuing concern as a component of commercial jet oils (115). It is bioactivated by P450 oxidation and then albumin-catalyzed cyclization of the hydroxymethylphenyl intermediate (116–118) (Figure 4). Studies of other OPs and the neurotoxicity mechanism emphasized hens, clearly the best model for OP-induced delayed neuropathy (OPIDN) in humans, because of similar 10–20 day delays from OP exposure to clinical signs and peripheral neuropathy (11, 114, 119). There are some similarities but also critical differences in the responses of mice as compared with hens and humans to OPIDN agents (114, 120). Several OPs that induce OPIDN in hens also induce a delayed toxicity in mice proceeding to death in 1–3 days and some compounds that enhance or retard OPIDN in hens do the same for OP-induced delayed toxicity in mice (120).

5.1.2. Discovery of the Target

DFP and mipafox (but not paraoxon) produce the same syndrome as TOCP in hens, which is completely unrelated to AChE inhibition (111, 119). The OPIDN target in hen brain was named NTE as the paraoxon (50 μM) resistant and mipafox (40 μM) sensitive portion, either of the enzymatic hydrolysis of phenyl phenylacetate or phenyl valerate (which mimic the structure of the TOCP metabolite) (Figure 4) or of the site phosphorylated by [³H]DFP (111, 119, 121, 122). M. K. Johnson, P. Glynn, and associates of the Medical Research Council Toxicology in the United Kingdom systematically, thoroughly, and very successfully followed this problem for several decades, leading to isolation, cloning, and sequencing of the target protein NTE (119). The expression of an active site region (referred to as NEST) made it possible to study the substrate specificity (123), showing preferential hydrolysis of lysolecithin as a candidate endogenous substrate (124). Mouse brain was then used to identify NTE

Table 3. Organophosphate Selectivity for AChE vs NTE-LysoPLA (in Vitro IC₅₀ Ratios from Most Selective for AChE to Most Selective for NTE-LysoPLA)

AChE selective	paraoxon (>4000) ^a
little selectivity	CPO (9), dichlorvos (6), profenofos (0.5), DFP (0.13)
NTE-LysoPLA selective	IDFP (3 × 10 ⁻⁴), OBDPO (3 × 10 ⁻⁴), EOPF (4 × 10 ⁻⁴), DSF (<5 × 10 ⁻³), MAFP (5 × 10 ⁻³)

^a NTE/AChE IC₅₀ ratio. All values for mouse brain.

as a LysoPLA (designated NTE-LysoPLA); that is, the same OP specificity and sensitivity are observed in vitro and in vivo as the paraoxon resistant and mipafox sensitive activity using either phenyl valerate or lysolecithin as the substrate (36). NTE-related proteins with ≥60% homology to NTE are found in the brain of mouse, rat, and human and function as an esterase in rats (125) but cannot substitute for NTE in mice to prevent neurotoxicity (28). Cells expressing high levels of NTE show deformation of the endoplasmic reticulum (126). NTE in mammalian cells and its homologue in yeast degrade phosphatidylcholine to glycerophosphocholine, thereby playing a central role in membrane lipid homeostasis (127).

5.1.3. Mechanistic Aspects

Lysolecithin is a major phospholipid in membranes, and elevated levels are associated with atherosclerosis, hyperlipidemia, inflammation, and lethal dysrhythmias in myocardial ischemia (128). It is also a signal transducer for G-protein-coupled actions (such as development of inflammatory autoimmune disease) via the immunoregulatory receptor G2A (129, 130). Lysolecithin is a classical demyelinating agent and has long been used for studying demyelination–remyelination. The potent NTE inhibitor EOPF at 3 mg/kg ip in mice significantly elevates the lysolecithin level in spinal cord (possibly related to localized demyelination) but not in frontal lobe or pons/medulla (131). LysoPLA is one of many phospholipases involved in phospholipid degradation and membrane remodeling. NTE-LysoPLA is more sensitive than other phospholipases studied to OP delayed toxicants (Table 2) (132, 133). NTE-LysoPLA (<30% of total LysoPLA activity in mouse brain) is also more sensitive than total LysoPLA, but OP structure–activity comparisons suggest active site similarities or identities (131). EOPF is a potent inhibitor of total LysoPLA activity (IC₅₀ = 2 nM) (131). The aging of dialkylphosphoryl-NTE-LysoPLA is proposed to give it a “toxic gain of function” as an important step in OPIDN (119), but this is not essential in OP-induced hyperactivity and delayed toxicity in mice (28, 120).

5.1.4. OP Structural Requirements

The inhibitory specificity of OPs for NTE-LysoPLA vs AChE (Tables 2 and 3) is a useful criterion in predicting OP action as OPIDN agents as compared with acute lethality (119). The OP NTE inhibitors were optimized with hens and mice, leading to EOPF and OBDPO as highly potent designer compounds with relatively low AChE effects as acute toxicants (120). The current OP insecticides have been tested for possible OPIDN in the hen NTE assay in vitro and in vivo with single or multiple doses (119). Some OPs considered to be OPIDN active are no longer used (e.g., mipafox and leptophos) or considered to be extremely hazardous (EPN) (for structures see ref 1). Leptophos was the causal agent in

a major incident of OPIDN in water buffalo (134). The remaining OP pesticides are OPIDN negative or only show as positive with massive and unrealistic doses, e.g., CP (11, 135).

5.1.5. Toxicological Relevance

Studies with *Drosophila*, hens, and mice are possibly relevant to the importance of NTE and NTE-LysoPLA in OP-induced delayed effects in humans. The identity of 41% between the sequence of NTE from human brain and the swiss cheese *sws* protein from *Drosophila* is of interest since the X-linked *sws* mutation causes glial hyperwrapping and brain degeneration in *Drosophila* (26, 136). Seventy to ninety percent inhibition is required for OPIDN in hens (119), and a similar value is required for delayed toxicity in mice (120). Importantly, the characteristic pathology observed in humans and hens is not pronounced in mice. However, mice are more appropriate for gene deletion to define the developmental and behavioral consequences of low NTE-LysoPLA activity (28). NTE of mouse brain was sequenced, and gene deficient mice were prepared with embryo lethality for *Nte*^{-/-} and hyperactivity of adults for *Nte*[±]. Embryonic lethality is attributed to placental failure and impaired vasculogenesis, suggesting that NTE-LysoPLA is required for normal blood vessel development (29). The same type of elevated motor activity observed over a 10 day period for *Nte*[±] mice is induced by EOPF at a single ip dose of 1 mg/kg (28). The *Nte*[±] mice had 55–59% of the NTE-LysoPLA activity of *Nte*^{+/+} littermates with either phenyl valerate or lysolecithin substrate (36). Brain specific deletion of *Nte* in mice results in neurodegeneration, indicating that a loss of function mechanism may contribute to vacuolation and neuronal loss (30). Thus, OPs that selectively inhibit NTE-LysoPLA relative to AChE in hen and human brain are candidate OPIDN agents and, in mice, may be inducers of hyperactivity. Further understanding of the species and compound specificities will help define the role, if any, of OP pesticides and chemical warfare agents in related toxicological problems of OPIDN, delayed toxicity, and hyperactivity. Testing procedures and policies for OPIDN have removed several OP pesticides from the market, and those remaining have a low risk factor coupled with a long history of safe use. Mechanistic knowledge has made it possible to evaluate NTE-LysoPLA-related risks and maintain appropriate restrictions.

5.1.6. Other Types of Delayed Effects

There are also other delayed effects probably not associated with NTE-LysoPLA inhibition. OPs used in sheep dips are possible inducers of chronic fatigue syndrome in the United Kingdom (137). Sarin is a proposed contributor to lingering health problems of combat troops in the Gulf War (138). A critical evaluation of data leading to these reports and other epidemiological and clinical studies gave no evidence of peripheral nerve dysfunction

caused by low-level prolonged exposures to OP insecticides or chemical warfare agents (139). The insecticide impurities $\text{CH}_3\text{SP}(\text{O})(\text{OCH}_3)_2$ and $(\text{CH}_3\text{S})_2\text{P}(\text{O})\text{OCH}_3$ and related compounds induce multiple organ toxicity and delayed death (140–142).

5.2. Signal Transduction and Gene Expression

Several signal transduction and gene expression systems are affected by OPs in vitro or in vivo (see also Section 4.3). CPO (5–50 μM) in CHOK1 cells induces ERK 44/42, which are also induced by diacylglycerol (e.g., 1,2-dihexanoylglycerol), with a greatly increased effect for CPO plus diacylglycerol; that is, when diacylglycerol lipase is inhibited (143, 144). CP (50 μM) induces apoptosis in cultured rat cortical neurons that is regulated by a balance between p38 and ERK/JNK MAP kinases (145). MAFP (10 μM) induces cyclooxygenase 2 expression in murine J774 macrophage cells via signal transduction involving PKC, ERKs, and p38 MAP kinase (146). DFP (1 μM) in mouse brain slices induces overexpression of mRNA encoding the transcription factor c-Fos, which is followed by increases in mRNA for AChE and decreases in mRNAs for ChAT and vAChT (147).

Some of the OP effects are also observed in vivo. CP and fenthion ($2 \times 0.25 \text{ LD}_{50}$, oral) are the most potent insecticide activators of PKC in rat brain and liver from a survey investigation, and at 50 μM , they also increase PKC in cultured neuronal PC-12 cells (148). CP (8 mg/kg oral in rats) increases P-glycoprotein expression, which may play a role in xenobiotic detoxification (149). Phenyl saligenin cyclic phosphate (2 mg/kg, im) alters the expression of α -tubulin transcripts in the spinal cords of treated hens (150). Sublethal doses of sarin (50 $\mu\text{g}/\text{kg}$, im) alter mRNA expression of α -tubulin, vimentin, and glial fibrillary acidic protein in the central nervous systems of rats (151, 152). CP (125 mg/kg, ip) alters many gene expression patterns in rats, but the specific enzymes and systems associated with these changes remain to be established (153). Rapid advances in signal transduction and gene expression methodology will continue to provide a better understanding of the events between initial OP target interactions and observed organismal effects.

6. Cannabinoid System

The endocannabinoid arachidonyl ethanolamide (anandamide) (Figure 5) and the endogenous sleep-inducing agent oleamide (Figure 5) are hydrolyzed by FAAH (154). Anandamide also binds to the CB1 receptor in brain, leading to antinociception, catalepsy, and suppression of spontaneous activity. The CB1 receptor is the target for marijuana and its principal psychoactive ingredient Δ^9 -tetrahydrocannabinol. Both the FAAH and the CB1 components of the cannabinoid system are sensitive to many OPs, including insecticides and their metabolites, and to some organosulfonyl fluorides.

6.1. FAAH

The duration of action of anandamide is limited by FAAH. FAAH gene deficient mice are severely impaired in their ability to degrade anandamide and, when treated with this compound, exhibit an array of intense CB1-

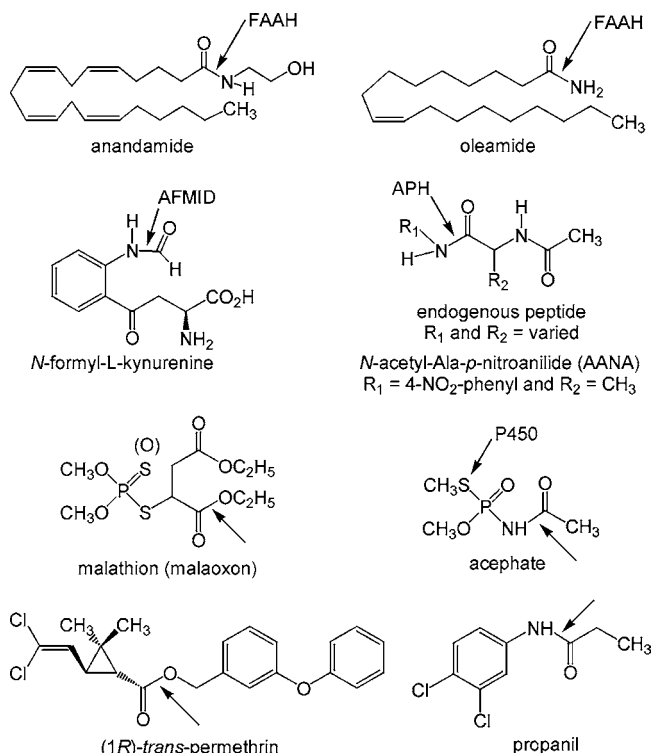


Figure 5. Substrates of pesticide-hydrolyzing carboxylesterases and amidases.

dependent behavioral responses, including hypomotility, analgesia, catalepsy, and hypothermia (20). The OP sensitivity of FAAH was first shown with MAFP and rat brain enzyme ($\text{IC}_{50} = 2.5 \text{ nM}$) (155). FAAH of mouse brain is very sensitive to MAFP, IDFP, arachidonylsulfonyl fluoride, and DSF ($\text{IC}_{50} = 0.1\text{--}2 \text{ nM}$) (37, 40). Some OP compounds prolong the action of exogenous anandamide in mice (34). A portion of the behavioral effects of cannabinoids in rats is reproduced by MAFP and methyl alkylfluorophosphonate analogues (156). Particularly potent inhibitors in vivo in mice are CPO and tribufos (34). The normal use patterns for OP pesticides are unlikely to cause any toxicity attributable to FAAH inhibition although several OPs are equally potent inhibitors of FAAH and NTE (35).

6.2. CB1 Receptor

A CB1 site(s), assayed as binding of the cannabinoid analogue [^3H]CP 55,940, is blocked by several OPs and alkanesulfonyl fluorides although the functional significance is unknown (155, 157). CB1 gene deficient mice appear healthy and fertile, but they have a significantly increased mortality rate (32). They also display reduced locomotor activity, increased ring catalepsy, and hypoalgesia in hotplate and formalin tests. Δ^9 -Tetrahydrocannabinol-induced ring catalepsy, hypomotility, and hypothermia are completely absent in CB1 deficient mice (32). The first report of a FAAH inhibitor also active on CB1 was that for MAFP with rat brain receptor ($\text{IC}_{50} = 20 \text{ nM}$) (155). IDFP and DSF are equipotent ($\text{IC}_{50} = 2\text{--}7 \text{ nM}$) for FAAH and CB1, but generally, FAAH is more sensitive (37, 39, 40). CPO is also a potent in vitro inhibitor of CB1 receptor binding ($\text{IC}_{50} = 14 \text{ nM}$) but is effective in vivo only at near lethal levels (38). Ex vivo studies with mouse brain indicate that OPs inhibit FAAH much more than CB1 and that these targets overall

appear to be less important in poisoning than AChE and NTE-LysoPLA (35, 39).

7. Other Serine Hydrolase Targets

7.1. AFMID

Many OPs and a few methylcarbamates are avian teratogens; that is, they induce severe developmental abnormalities including malformation of the lower extremities (micromelia) and abnormal feathering when injected into chicken eggs at days 4–6 of incubation (158, 159). The biochemical target was defined by three observations in chicken eggs (159). First, nicotinic acid, nicotinamide, and metabolic precursors thereof partially to completely protect against or ameliorate the abnormalities (158, 160). Second, the severity of teratogenic signs is correlated with the lowering of embryo NAD level (161, 162). Finally, the primary lesion is inhibition of AFMID (formerly known as kynurenine formamidase) (163, 164) (Figure 5). The OP or methylcarbamate creates a nicotinic acid deficiency by blocking its biosynthetic pathway. Tryptophan is the metabolic precursor for *N*-formyl-L-kynurenine, L-kynurenine, nicotinic acid, NAD(H), and NADP(H) in mammals. AFMID, the second enzyme in this pathway, is very sensitive in birds and mammals to many OPs and methylcarbamates. Highly potent teratogens include the OPs diazinon, diazoxon, and monocrotophos and the methylcarbamates physostigmine and carbaryl active at 10–300 $\mu\text{g}/\text{egg}$ (158–160, 162). The most potent inhibitors (and teratogens) are OPs with nitrogen-containing heterocyclic or aliphatic leaving groups (165). Other OPs including parathion induce vertebrate malformations such as brevicollis due to embryo AChE inhibition (159). The inhibitor specificity for AFMID is somewhat similar to yet distinct from that for AChE (159, 166). OP teratogenesis is much more prominent in the bird than the mammal, in part because embryogenesis in the bird requires yolk tryptophan conversion to nicotinic acid in contrast to a dietary source for the vitamin in mammals (159). There are also some diazinon-induced changes in the metabolism of tryptophan in mammals (159, 167, 168). An *Afmid* deficient mouse will help further define the ramifications of reduced AFMID activity in mammals (169).

7.2. APH

APH hydrolyzes the N-terminal acetylated amino acid residue on peptides (Figure 5), e.g., α -melanocyte-stimulating hormone, thereby removing one form of protection from proteolysis (170). It also hydrolyzes oxidized proteins in human erythrocytes with a possible physiological function of removing oxidatively damaged components in cells (171). APH is the most sensitive enzyme in rat brain to phosphorylation by [³H]DFP (42) and is also identified by radiolabeling with a biotinylated OP probe (172). APH activity is potently inhibited by three OPs (CP methyl oxon, dichlorvos, and DFP) with $\text{IC}_{50} = 18\text{--}119\text{ nM}$, i.e., 6–10-fold greater sensitivity as compared to AChE (42). APH is not an alternate target for OP acute lethality since <40% is inhibited in *Ache*^{-/-} mice severely poisoned with VX ($\text{LD}_{50} = 10\text{--}12\ \mu\text{g}/\text{kg}$) (59). However, it is a possible target of pharmacological significance for cognitive-enhancing drugs such as the OP metrifonate (Figure

2) (173). The consequences of long-term in vivo inhibition remain unknown, but the ubiquity of *N*-acetyl proteins [e.g., >50% of those in cytosol (174)] suggests a likelihood of organismal effects.

7.3. Carboxylesterases and Amidases

7.3.1. OP Inhibitors as Modifiers of Non-OP Toxicity

Pesticides or pharmaceuticals detoxified or activated by carboxylesterases and amidases (175) may be altered in their toxicity by OP or methylcarbamate inhibitors of these enzymes. This can be intentional on use of mixtures to enhance potency or inadvertent on multiple exposures. The pyrethroid insecticides [e.g., (1*R*)-*trans*-permethrin] (Figure 5) are hydrolyzed by “pyrethroid esterases,” more rapidly for *trans* than *cis* acid moieties and primary than secondary alcohol esters, and these enzymes in all species are OP sensitive (176–178). The insecticidal activity of several pyrethroids can be increased or synergized, sometimes by 3–68-fold, by adding a detoxification-inhibiting OP but perhaps increasing the mammalian toxicity as well (176–178). Insecticide-hydrolyzing esterases are generally better characterized in insects relative to resistance than in mammals. Resistance to OP insecticides in several insect species is associated with changes in carboxylesterase activity, and in one case, a single amino acid substitution can convert a carboxylesterase to an OP hydrolase, thereby conferring insecticide resistance (179). Propanil (Figure 5) is detoxified by an amidase in rice, providing a safety factor which is lost when the crop is exposed to OP or methylcarbamate amidase inhibitors, used as insecticides, resulting in crop injury (180). Carboxylesterases can act as scavengers to protect against OPs, facilitated by spontaneous reactivation after OP inhibition, with greater importance for highly toxic compounds (e.g., sarin, soman, tabun, and paraoxon) than for less toxic ones such as DFP (181–184). The amidase hydrolyzing the antidepressant isocarboxazid is inhibited by the OP pesticide fenitrothion at 0.5 mg/kg in rats (185).

7.3.2. OP as Both Serine Hydrolase Substrate and Inhibitor

A single OP pesticide may be both a substrate and an inhibitor for OP sensitive serine hydrolases as illustrated here with malathion and acephate (Figure 5). In the classical case, the selectivity of malathion depends in large part on preferential detoxification by carboethoxy hydrolysis in mammals and activation by P450-catalyzed oxidative desulfuration in insects; as an additional control feature, malaoxon as the activation product inhibits the detoxifying carboxylesterase (186, 187). In an interesting variation, acephate is activated first by an amidase and second by an oxidase, presumably yielding methamidophos sulfoxide, which inhibits the amidase to stop further activation in mammals but apparently not insects (188). Malathion and acephate therefore inhibit their own metabolism either in chronic feeding studies or following a single high dose, decreasing the carboxylesterase detoxification of malathion and amidase activation of acephate. Another form of pesticide interaction is the synergism in toxicity from dual exposure to two OPs, one serving as the substrate (or compound potentiated) and

the other the inhibitor for carboxylesterases and amidases (189).

7.4. Identification of OP-Phosphorylated Proteins

One approach to identify other serine hydrolase targets is to label a tissue homogenate or fraction with a radioactive OP and then isolate and identify the labeled proteins and relate their labeling or inhibition of enzymatic activity to the toxic dose from *ex vivo* studies. [³H]DFP radiolabels 24 proteins in rat brain homogenate, but the one most extensively labeled is an APH identified by sequencing tryptic peptides (42, 190, 191). Similarly, [³H]DFP allowed identification of a phospholipase secreted by activated platelets and APH in human erythrocytes (171, 192). Another approach is to isolate the proteins that are derivatized with a specific biotinylated OP probe using an avidin matrix (193, 194). This was a critical procedure in NTE isolation and identification (Figure 4) (195). It also allowed identification of biotinylated APH and another protein in rat brain homogenate (194). A limitation to this procedure is that most OP toxicants have short alkyl chains while the biotinylated probe *a priori* is a larger molecule with a spacer unit, which may greatly change the specificity and reduce the inhibitor potency. A third, and less definitive, approach is to define the pharmacological profile of [³H]DFP labeling using endogenous compounds (196) or unlabeled OPs (190) as competitors for the site undergoing phosphorylation.

7.5. Other Candidate Targets

There are also additional OP targets *in vitro* or *in vivo* of varied or unknown toxicological relevance. Four of them are reported to be particularly sensitive. Malathion and malaoxon (IC₅₀ = 1–9 nM) inhibit lysyl oxidase in homogenates of *Xenopus* embryos, suggesting that they alter posttranslational modification of collagen with resultant morphological defects in connective tissue (197). CP and CPO at below 1 nM are reported to activate Ca²⁺/cAMP response element binding protein (also known as CREB) in cultured rat neurons as a possible mechanism for neurotoxicity (198). Paraaxon (1–10 nM) causes apoptotic cell death in a leukemia cell line by disruption of mitochondria, leading to activation of caspase-9 (199). MAFP at <1 μM and DFP at 100 μM inhibit platelet-activating factor acetylhydrolase, and the purified enzyme from bovine brain is labeled by [³H]DFP (200, 201). Fenitrothion at 22 nM acts as an androgen receptor antagonist *in vitro* (202) and inhibits the development of androgen-dependent tissues *in vivo* (15 mg/kg/day) with a potency comparable to the pharmaceutical anti-androgen flutamide (203).

Other *in vitro* systems are much less sensitive. ATPases are inhibited by high micromolar to millimolar levels of OPs, *i.e.*, CPO for Ca²⁺-stimulated ATPase (204) and paraaxon for Na⁺/K⁺-ATPase (205). Blood clotting factors (thrombin, plasmin, and kallikrein) and digestive enzymes (α-chymotrypsin, trypsin, and elastase) are of moderate to low OP sensitivity (206, 207). CP (2 μM) (but not CPO) increases reactive oxygen species in PC12 cells (208). The genotoxicity of numerous OP insecticides has been evaluated (209). The mutagenicity of dichlorvos to bacteria is due to methylation of DNA, which does not

occur with mammalian DNA under realistic exposure conditions (210). CP and CPO (30 μM) inhibit DNA synthesis in cultured cells (211). CP and its trichloropyridinol hydrolysis product at 3 μM both inhibit neuronal differentiation in PC12 cells by a mechanism independent of AChE inhibition (212). Neurite outgrowth is inhibited by mipafox and OBDPO at 100 μM in PC12 and C6 cells (213) and by mipafox (but not paraoxon) at 1 μM in dorsal root ganglia cell cultures (214) or 50 μM in SH-SY5Y cells (215). In contrast, paraoxon (but not mipafox) (50 μM) alters Ca²⁺ homeostasis in these differentiated SH-SY5Y cells (215). Parathion and TOCP at 100 μM to 1 mM inhibit the mitochondrial transmembrane potential as a possible target for cytotoxicity in cultured cells (216). Several OP pesticides, both phosphates and phosphorothionates (IC₅₀ ca. 10 μM), displace binding at the *N*-methyl-D-aspartate receptor of rat brain (217). Dichlorvos (IC₅₀ = 0.3–2 mM) decreases the activities of murine splenic natural killer, cytotoxic T lymphocyte, and lymphokine-activated killer by a proposed mechanism involving serine protease (granzyme) inhibition (218).

Developmental neurotoxicity (219) and numerous *in vivo* effects have been reported for CP in rats: mitotic abnormalities and apoptosis are elicited at 1 μM in the neuroepithelium of cultured rat embryos (220); developing rats treated *sc* have changes in neurospecific proteins (221) and altered serotonin receptors, transporter, and associated signal transduction (222); repeated low-dose exposure (2.5–25 mg/kg *sc* daily) leads to growth retardation, behavioral abnormalities, and muscle weakness attributed to effects on axonal transport (223); a 45 mg/kg *sc* dose reduces brain catalase 28% in rats (224); and a 2 mg/kg *sc* dose inhibits DNA synthesis by up to 26% in all brain regions within 4 h for neonatal rats (225). DFP (1.5 mg/kg, *sc*) induces hyperthermia in rats by a pathway possibly involving prostaglandin release (226). Myotoxicity during DFP-induced (1.5 mg/kg, *sc*) rat muscle hyperactivity is suggested to arise from depletion of high-energy phosphates by a mechanism involving nitric oxide and oxidative stress (227). OP pesticides elicit a variety of immunotoxic effects in laboratory animals, wildlife, and humans through both cholinergic and non-cholinergic pathways (141, 228).

8. Summary

OPs have been the major insecticides in number and market share for many decades, and they are still essential tools in crop protection and public health (229). They were the first highly effective systemics moving throughout plants to protect even the growing tip from sucking insect pests for several days or weeks. Their selective toxicity is based on specificity differences in the AChE targets, more rapid detoxification in mammals than insects, and the use of proinsecticides undergoing preferential activation in insects as compared with mammals. Their facile biodegradation and low environmental persistence are coupled with toxic effects more likely due to acute rather than chronic exposure. The OPs have been more extensively studied than any other class of pesticides (10, 230, 231), and the risks are summated for all AChE inhibitors (232). Great care has been taken to ensure their safe use based on AChE as the primary target.

Safety evaluations in OP toxicology have evolved from focusing on AChE as the single most important target

to consideration of several OP sensitive serine hydrolases and receptors as secondary targets. The toxicological relevance of each of the main targets considered here depends on its sensitivity to inhibition *in vitro* and *in vivo*, the physiological significance of the disruption or lesion, and the level of exposure and degree of inhibition that take place under actual use conditions. The mechanism studies often rely on designer compounds of outstanding potency and selectivity to accentuate specific phenomena for ease of evaluation. High-dose laboratory experiments with animal models (e.g., mice, rats, and chickens) are difficult to relate to low-dose, long-term environmental exposure and particularly to actual risks for people. The findings reviewed reconfirm the importance of AChE as the primary target and NTE-LysoPLA as the secondary target of greatest interest (Figure 1). The safety of the continued use of OPs in agriculture and potential expanded use in medicine depends on understanding the relevance not only of AChE inhibition but also of these secondary targets in the health effects of acute and long-term exposures.

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